CD3⁻ Large Granular Lymphocytes Recognize a Heat-Inducible Immunogenic Determinant Associated With the 72-kD Heat Shock Protein on Human Sarcoma Cells

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Traditionally, heat shock proteins (HSPs) are believed to be located intracellularly, where they perform a variety of chaperoning functions. Recently, evidence has accumulated that some tumor cells express HSPs on the cell surface. The present study confirms this finding and correlates HSP72 cell surface expression, induced by nonlethal heat shock, with an increased sensitivity to interleukin-2–stimulated CD3⁻ natural killer (NK) cells. After nonlethal heat shock, a monoclonal antibody directed against the major heat-inducible 72-kD HSP (HSP72) stains the cell surface of sarcoma cells (ie, Ewing's sarcoma cells or osteosarcoma cells) but not that of normal cells (ie, peripheral blood lymphocytes, fibroblasts, phytohemagglutin-stimulated blasts, B-lymphoblastoid cell lines) or of mammary carcinoma cell line MX-1 carcinoma cells. In this study, we show for the first time a correlation of HSP72 cell surface expression with an increased susceptibility to lysis by NK effector cells. This finding is supported by the following points: (1) HLA-disparate effector cells show similar, elevated lysis of HSP72⁺ heat-treated sarcoma cells; (2) CD3⁻ NK cells, but not CD3⁺ cytotoxic T lymphocytes, are responsible for the recognition of heat-shocked sarcoma cells; (3) by antibody-blocking studies, an immunogenic HSP72 determinant, which is expressed selectively on the cell surface of heat-treated sarcoma cells could be correlated with NK recognition; (4) the reported phenomenon is independent of a heat-induced, transient downregulation of major histocompatibility complex (MHC) class-I expression; and (5) blocking of MHC class-I–restricted recognition, using either MHC class-I–specific monoclonal antibody W6/32 on the target cells or α/β T-cell receptor monoclonal antibody WT31 on effector cells, also has no inhibitory effect on the lysis of HSP72⁺ tumor cells. Finally, our in vitro data might have further clinical implications with respect to HSP72 as a stress-inducible, sarcoma-specific NK recognition structure.

HEAT, LIKE OTHER STRESS factors, induces an increased synthesis of heat shock proteins (HSPs), especially of the highly stress-inducible 72-kD HSP (HSP72), a member of the HSP70 family.[1-4] Intracellularly, HSPs perform a variety of chaperoning functions that help to maintain the cytoskeletal integrity and metabolic homeostasis of cells under stress conditions.[5,6] Members of the HSP70 family, HSP72 and HSP73, respectively,[7,8] are known to play a role in the intracellular translocation, assembly, and disassembly of other proteins[9-13] and have been shown to bind tumor-specific peptides[14] in an adenosine triphoshate-sensitive mode.[15,16] Recently, evidence has accumulated that HSPs are also localized on the cell surface.[17-21] The peptide-binding protein that shows high homology to members of the HSP70 family is found on the cell surface in association with major histocompatibility complex (MHC) class-II molecules.[17] Cell surface localization of HSP72, the heat-inducible form of HSP70, was found on affected retroocular fibroblasts derived from patients suffering from Graves' ophthalmopathy[18] and on virus-infected cells,[19] but not on normal fibroblasts.[18] In BALB/c mice, methylcholanthrene-induced sarcoma cells also express HSP70 on the cell surface.[20] Recent data from our group[21] showed a heat-inducible cell surface expression of HSP72 on different human tumor cell lines. Although HSPs are among the most highly conserved proteins[22,23] with a wide phylogenetic representation,[24] HSPs have been found to act as specific immunogenic determinants expressed on tumor cells.[25-27] Thus, the question arises as to whether HSPs themselves or HSP-associated peptides act as immunogenic determinants. Recently, immunogenic peptides were found to be associated with HSP70,[28] which can be recognized by T cells. In the present report, we addressed the question as to whether interleukin-2 (IL-2)–stimulated natural killer (NK) cells, which are known to play an important role in the elimination of tumors, might also play a role in the recognition of HSPs. The oncolytic function of NK cells can be stimulated by the loss of certain MHC class-I alleles on tumors[29-31] and can be further enhanced by activation with different cytokines, predominantly with IL-2.[32,33] Here, we show that nonlethal heat shock selectively induces a cell surface expression of an immunogenic HSP72 determinant on human sarcoma cells that persists for at least 96 hours. Furthermore, heat leads to a transient decrease in the MHC class-I expression. Functionally, we show that heat shock leads to an increased susceptibility to lysis by non-MHC–restricted, CD3⁻ NK cells. This phenomenon could be correlated with the heat-inducible cell surface expression of the immunogenic HSP72 determinant[21] but is not because of a decrease in the MHC class-I expression.

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MATERIALS AND METHODS

All cell lines were screened (Mycoplasma tissue culture Genprobe; H. Biermann, Bad Nauheim, Germany) and defined as negative for mycoplasma contaminations.

Peripheral blood lymphocyte (PBL) preparations and generation of Epstein-Barr virus (EBV)-transformed B-lymphoblastoid cell lines

(B-LCLs). PB obtained from four different healthy human volunteers (A, B, C, and D) was anticoagulated with heparin (Heparin Novo; Novo Nordisk Pharma GmbH, Mainz, Germany). PB mononuclear cells were separated by Ficoll Isopaque (Ficoll Paque; Pharmacia, Uppsala, Sweden) density gradient centrifugation. After separation, PBLs were obtained and incubated in RPMI 1640 (GIBCO, Eggenstein, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS; GIBCO), 6 mmol/L L-glutamine (GIBCO), and antibiotics (100 IU/mL penicillin and 100 μg/mL streptomycin; GIBCO). Following the established method of Terasaki and McClelland,[34] PBLs derived from healthy human blood donors (A, B, C, and D) were HLA-typed with defined alloantisera and monoclonal HLA-specific antibodies as follows: Donor A: A2, B60(40), B62(15), Bw6, Cw3, DR4, DR13(6), DQ6(1), DQ7(3), DR52/53; Donor B: A2, A29(19), B44(12), B61(40), Bw4, Bw6, Cw2, Cw4, DR7, DQ1, DQw7(3); Donor C: A3, A11, B35, B39(16), Bw6, Cw4, DR1, DR3, DR52, DQ1, DQ2; and Donor D: A24(9), A30(19), B7, B27, Bw4, Bw6, Cw2, Cw7.

A human EBV-transformed B-LCL was established from freshly isolated PBLs derived from donor D. Briefly, PBLs (10×10^6) were incubated in 5 mL RPMI 1640 medium containing 25% FCS, 6 mmol/L L-glutamine, antibiotics (for concentrations, see above), and an equal volume of EBV-containing supernatant (from the EBV producer cell line B95-8; American Type Culture Collection [ATCC], Rockville, MD) for 10 to 14 days. Phytohemagglutinin (PHA-M; Difco, Hamburg, Germany) was added to a final concentration of 1%. After 3 weeks, a permanently growing EBV-transformed B-LCL was generated.

Cell culture of normal and malignant cells. Allogeneic EBV-transformed B-LCLs, K562 cells (a myeloid tumor cell line derived from a patient with chronic myelogenous leukemia in blast phase; ATCC, CCL 243),[35] the osteosarcoma cell lines HOS58 (kindly provided by Dr Schmidt, GSF-Institute for Molecular Virology) and MG63 (ATCC, CRL 1427), and the mammary carcinoma cell line MX-1 (kindly provided by Dr Wagner, University of Lübeck and DKFZ Heidelberg, Institute for Experimental Pathology) were grown in RPMI 1640, supplemented with 10% FCS, 6 mmol/L L-glutamine, penicillin, and streptomycin (for concentrations, see above).

Exponentially growing 5838 Ewing's sarcoma (ES) cells, derived from a patient suffering from ES,[36] kindly provided by Dr M.L. Meltz (University of Texas, San Antonio, TX), were maintained in RPMI 1640, supplemented with 15% FCS, 6 mmol/L L-glutamine, 0.01 mol/L NaOH (pH 8.5 is essential for growth of tumor cells), and antibiotics (60 IU/mL penicillin and 60μg/mL streptomycin). As growth parameters, plating efficiency (PE) and multiplicity (N) of these cells under constant culture conditions at 37°C were defined as PE equals 40% to 60% and N equals 2.0 to 2.2. HLA typing of ES cells following the Terasaki method was not possible. Therefore, ES cells were typed by flow cytometry as described below in the Heat Treatment section.

Clonogenic cell surviving assay. Exponentially growing monolayer cells were treated at different temperatures (41.8°C, 42°C, 43°C, 44°C, and 45°C) for varying incubation periods ranging from 0 to 450 minutes in a temperature-controlled waterbath (Haake E3, Karlsruhe, Germany). After treatment, the cells were trypsinized, counted, and plated at dilutions of known cell numbers (10^2 to 10^5 cells/flask) into four replicate flasks. After about 12 days, the colony number was determined, and the cell viability was calculated with corrections for the PE. In addition, cell viability was also tested by using dye exclusion assays (trypan blue, propidium iodide). The temperature of 41.8°C was defined as nonlethal for human tumor and normal cells because, at this temperature, greater than 97% of the cells remained viable in the clonogenic surviving assay[21] and more than 98% of the cells excluded trypan blue and propidium iodide.

Heat treatment. Exponentially growing cells were treated with the nonlethal temperature (41.8°C) for 200 minutes in a temperature-controlled waterbath (Haake E3) and were then incubated at 37°C for different time intervals ranging from 0 to 96 hours. The heat dose parameter of 41.8°C was chosen in accordance with data obtained from clonogenic cell surviving assays.[21]

Monoclonal antibodies (MoAbs), indirect immunofluorescence, and FACScan analysis. Phenotypic characterization of viable effector and target cells was performed by indirect immunofluorescence followed by flowcytometric analysis on a FACScan instrument (Becton Dickinson and Co, registered trademark for a fluorescence-activated cell sorter, Heidelberg, Germany). Viable cells (1×10^6) were incubated with the following antibodies (final concentration, 5 $\mu g/1 \times 10^6$) each containing 0.1% sodium azide (NaN_3) at 4°C for 1 hour. The isotype and the specificity of the antibodies is given in parentheses: anti-HSP27 (IgG1), an isotype matched control MoAb for HSP72 (Dianova, Hamburg, Germany); anti-HSP72 (IgG1, RPN1197; Amersham, Braunschweig, Germany); mouse IgG2a isotype-matched control antibody for W6/32 (Dianova), W6/32 (IgG2a; anti-MHC class I); L243 (IgG2a; anti-HLA DR region); OKT3 (IgG2a, anti-CD3) MoAb kindly provided by Dr J. Johnson (München); anti-CD4 (IgG1; Dianova); anti-CD8 (IgG1; Dianova); anti-CD16 (IgG2a; Dianova); anti-CD19 (IgG1; Dianova); anti-CD56 (IgG1; Dianova); anti-CD57 (IgM; Dianova); WT31, an anti-α/β TCR (IgG1; Becton Dickinson); and δ-TCS1, an anti-γ/δ TCR (IgG1; T Cell Sciences, Cambridge, MA). For HLA typing of ES cells, monoclonal and polyclonal antibodies and antisera directed against the following epitopes were used: HLA-A2, A3, A9, A10, A25, A29, A30, A31, A32, and the entire A-region; HLA-B7, B8, B13, B14, B15, B17, B21, B22, B23, B27, B37, B44, Bw4, Bw6; HLA-Cw2, Cw3, Cw4, Cw7; HLA-DR1, DR3, DR5, DR6, DR7, DR8, DR12, DR13, DRw52; and HLA-DQ1, DQ2, DQ3, DQ5, DQ6, DQ7, DQ8, DQ9. After washing twice in phosphate-buffered saline/10% FCS, the cells were stained with a second fluorescein isothiocyanate-conjugated rabbit antimouse Ig antibody (DAKO, Hamburg, Germany) for 1 hour at 4°C. Quantitative flow cytometry was performed on a FACScan instrument. The percentage of positively stained cells was defined as the difference of the number of specifically stained cells minus the number of cells stained with the isotype-matched control MoAbs (see above). The data obtained from FACScan analysis represent the mean values of four independent experiments.

Generation of cytotoxic effector cells. Cytotoxic effector cells were generated by incubation of PBLs (50×10^6) from HLA-disparate donors (A, B, and C) with irradiated (60 Gy), heat-treated ES cells (50×10^6) in the presence of recombinant IL-2 (100 IU/mL; EuroCetus, Frankfurt, Germany) for 7 days. For heat treatment of tumor cells, viable ES monolayer cells were heat-treated at nonlethal temperatures (41.8°C for 200 minutes) in a temperature-controlled waterbath.

Separation of effector cell subpopulations. Briefly, PB mononuclear cells were separated into a $CD3^+$ T-cell (α/β and γ/δ T-cell receptor [TCR]-positive T cells) and a $CD3^-$ ($CD56^+$, $CD57^+$ NK cells) effector cell population by adherence selection,[37] followed by a magnetic bead separation method (Dynabeads M-450; Dynal, Hamburg, Germany) on day 7 after stimulation and expansion with recombinant IL-2 (100 IU/mL). For this purpose, both cell fractions (50 to 100×10^6) generated from PBLs of donor A or B were incubated with a CD3-specific MoAb (OKT3) at 4°C for 1 hour. After being washed twice with phosphate-buffered saline/5% FCS, the cells were incubated with 3 magnetic beads per cell (Dynabeads M-450), which were prewashed twice in RPMI 1640/5% FCS medium and then suspended in 0.5 mL RPMI 1640 medium. After 1 hour of incubation at 4°C, the cells were washed 3 times with 2 mL RPMI 1640 medium and then separated into two cell fractions by magnetic bead separation using the magnetic particle concentrator Dynal-MPC1 (Dynal). $CD3^+$ T cells were bound to the magnetic

beads, whereas $CD3^-$ cells remained in the supernatant. The magnetic beads were removed from the positively enriched cell population by dissociation using Detach-A-Bead solution (Dynal). After 4 washing steps and an overnight recovery period in RPMI 1640/15% FCS at 37°C, both cell populations were used separately in a cell-mediated lympholysis assay (CML). The phenotype characterization of both cell fractions was performed by FACScan analysis using MoAbs directed against CD19 (IgG1), CD3 (IgG2a), γ/δ TCR (IgG1), CD16 (IgG2a), CD56 (IgG1), and CD57 (IgM). The percentage of γ/δ TCR^+ T cells was less than 5%; no $CD19^+$ B cells were found. Further phenotypic characterization is given in the figure legends.

Cytotoxicity assay. The specificity of IL-2–stimulated effector cells was monitored in a standard ^{51}Cr release assay.[38] The following cells were used as ^{51}Cr-labeled (5 mCi/mL stock; 0.4 mCi in 0.2 mL RPMI 1640/1 $\times$ 10^6 cells; NEN-DuPont, Bad Homburg, Germany) target cells (3,000/well), either untreated (37°C) or heat-treated at nonlethal temperatures (41.8°C for 200 minutes, followed by a 4-hour or 12-hour recovery period at 37°C): ES cells, K562 cells, EBV-transformed B-LCLs, and MX-1 cells. After a 2-hour labeling period, the target cells were washed 3 times and adjusted to 10^4 cells/mL in RPMI 1640/15% FCS medium (CML medium). The cells were coincubated at 37°C for 4 hours in 96-well round-bottomed microtiter plates (Greiner, Nürtingen, Germany) in a total volume of 0.2 mL/well, with cytotoxic effector cells at varying effector-to-target (E/T) ratios. The percentage of specific lysis was calculated as: [(experimental release − spontaneous release) ÷ (maximum release − spontaneous release)] $\times$ 100. Spontaneous release (SR) was assessed by incubating target cells in the absence of effector cells. SR in all experiments was below 20%.

Antibody-blocking assay. Inhibition of lysis by MoAbs directed against antigens expressed on either target (anti-MHC class I, W6/32[39,40]; anti-HSP72, RPN1197) or effector cells (anti-α/β TCR, WT31)[41] was performed by preincubation of the cells in 0.1-mL aliquots for 2 hours at room temperature. As a control, the isotype-matched MoAb HSP27 was used. The inhibition assays were performed at a final concentration of $10\mu g/1 \times 10^6$ for each antibody. After two washing steps of the antibody-treated cells in CML medium, the cytotoxicity assay was performed as described above.

RESULTS

Heat treatment of sarcoma cells leads to HSP72 cell surface expression. A clonogenic cell surviving assay was performed to define nonlethal heat dose parameters for adherent cells. At a temperature of 41.8°C, the cell viability as well as the capacity for clonogenic cell growth of all tested tumor cell lines (ES, HOS58, MG63, and MX-1) and of normal fibroblasts was more than 98% even after heating periods greater than 450 minutes. In contrast, heat exposure of cells at temperatures above 42°C reduced the clonogenic cell viability of neoplastic and normal cells.[21] The viability of non-adherent cells (PBLs, B-LCLs, and PHA blasts) at 41.8°C (200 minutes) was more than 97%, as determined by trypan blue exclusion. For all further investigations, the nonlethal temperature of 41.8°C was used. Although total protein synthesis is reduced after heat shock, synthesis of HSP72, the major heat-inducible form of HSP70, is strongly induced. Indirect immunofluorescence studies were performed to compare cell surface expression of different molecules on either untreated or heat-treated cells. The results of a comparative study on cell surface expression of HSP72 and MHC class-I molecules on neoplastic human cell types (ES, HOS58, MG63 sarcoma cells, and MX-1 carcinoma cells) and on cells derived from healthy human individuals (PBLs, fibroblasts, PHA blasts, and B-LCLs) before and after nonlethal heat treatment are summarized in Table 1. Untreated tumor and normal cells show no significant HSP72 cell surface staining. After single nonlethal heat shock (41.8°C for 200 min) and a recovery period at 37°C (4 hours) up to one third of viable sarcoma cells was positively stained using HSP72 MoAb. In contrast, MX-1 carcinoma cells and cells derived from healthy human individuals (PBLs, fibroblasts, PHA blasts, and B-LCLs) showed no HSP72 cell surface expression under these heating conditions. Recently, we showed that coincubation of untreated sarcoma cells with HSP72 containing supernatants of lethally heat-shocked cells does not result in an unspecific HSP72 cell surface expression.[21] Therefore, adventitious deposition of HSP72 released by dead cells that nonspecifically binds onto live cells is very unlikely. A transient downregulation in the percentage and the fluorescence intensity of MHC class-I molecules after heat shock was observed only with tumor cells, not with normal cells. Kinetic data of flowcytometric analysis indicate that the sarcoma-specific HSP72 cell surface expression peaks 4 hours after nonlethal heat shock and persists for at least 96 hours (Table 2). A transient reduction in the MHC class-I expression selectively on sarcoma cells is observed between 3 and 6 hours after nonlethal heat shock. After a 12-hour recovery period at 37°C, the MHC class-I expression reaches its initial levels. Similar data were obtained with other sarcoma cells such as HOS58 and MG63 (data not shown). With respect to these findings, the functional assays were performed after nonlethal heat shock, followed by a 4-hour and 12-hour recovery period at 37°C.

Heat-induced increased susceptibility to lysis of sarcoma cells is mediated by HLA-disparate effector cells. The ES tumor cells were HLA-typed by flow cytometry as A25(10), A29(19), and DQ7(3). No HLA-DR (using L243 MoAb), HLA-B (using MoAbs directed against Bw4 or Bw6), or HLA-C (using 4 different MoAbs directed against HLA-C alleles) reactivity was found. Cytotoxic activity of HLA-disparate effector cells was tested in a CML assay against either untreated (37°C) or heat-treated (41.8°C for 200 minutes and 37°C for 4 hours) ES cells as tumor target cells. Donors A, B, and C were chosen to generate cytotoxic effector cells because they share one [DQ7(3) is shared between effector cells A and ES target cells], two [DQ7(3) and A29(19) is shared between effector cells B and ES target cells], or no [no HLA allele is shared between effector cells C and ES target cells] HLA alleles with the ES target cells. Figure 1 shows for all three HLA-disparate effector cell populations (A, B, and C) comparable lysis patterns. The lysis against nonlethally heat-treated (41.8°C for 200 minutes and 37°C for 4 hours) ES cells compared with that for untreated (37°C) ES cells was increased more than twofold. In three independent experiments using identical experimental parameters, lysis of heat-treated ES cells was always significantly stronger compared with that of untreated ES cells.

Lysis of heat-treated sarcoma cells is mediated by $CD3^-$ NK cells. With respect to the increased susceptibility of heat-shocked sarcoma cells by HLA-disparate effector cells, we investigated the question of whether this cytotoxic response is MHC-independent. Using the immunomagnetic

Table 1. Comparative Flow Cytometric Analysis of HSP27 (Isotype-Matched Negative Control Antibody for HSP72, IgG1), HSP72 (IgG1), and MHC Class-I (W6/32 MoAb, IgG2a) Cell Surface Expression on Neoplastic Versus Normal Cells Either Untreated (37°C) or Heat-Shocked at Nonlethal Temperatures

	MoAbs					
	HSP27		HSP72		W6/32	
	37°C	41.8°C*	37°C	41.8°C*	37°C	41.8°C*
Neoplastic cells						
ES	1.5 ± 0.7 (24)	1.2 ± 0.8 (26)	3.2 ± 0.8 (31)	31.4 ± 1.9 (117)†	83.7 ± 3.5 (123)	70.5 ± 2.8 (94)†
HOS58	1.8 ± 0.3 (19)	2.1 ± 0.5 (19)	2.3 ± 0.4 (26)	25.9 ± 3.9 (90)†	73.2 ± 2.9 (183)	54.1 ± 3.2 (143)†
MG63	1.6 ± 0.6 (11)	1.9 ± 0.4 (11)	1.9 ± 0.6 (15)	15.7 ± 3.0 (52)†	90.0 ± 4.1 (201)	78.4 ± 3.3 (172)†
MX-1	2.3 ± 0.5 (10)	2.2 ± 0.5 (12)	2.8 ± 0.4 (12)	2.3 ± 0.6 (12)	99.8 ± 2.4 (126)	99.2 ± 2.7 (87)
Normal cells						
PBL	0.3 ± 0.1 (11)	0.2 ± 0.1 (10)	0.0 ± 0.0 (10)	0.0 ± 0.0 (14)	92.7 ± 3.2 (231)	90.8 ± 4.0 (238)
Fibro	1.1 ± 0.3 (11)	1.0 ± 0.2 (12)	1.2 ± 0.1 (12)	2.2 ± 0.2 (11)	96.3 ± 3.7 (203)	97.4 ± 2.9 (251)
PHA	0.3 ± 0.1 (10)	0.2 ± 0.1 (10)	0.3 ± 0.1 (9)	0.3 ± 0.1 (10)	94.8 ± 2.9 (272)	95.5 ± 3.2 (259)
B-LCL	0.3 ± 0.2 (12)	0.4 ± 0.1 (13)	0.3 ± 0.1 (16)	0.3 ± 0.1 (13)	97.9 ± 4.3 (294)	96.0 ± 2.9 (307)

Values shown are the percentage of positively stained cells ± standard deviation (SD) with the average mean fluorescence intensity in parentheses. A mouse Ig2a isotype-matched control antibody was used for MHC class-I expression (data not shown). Neoplastic cells included ES cells (ES), osteosarcoma cells (HOS58, MG63), and Mamma carcinoma cells (MX-1). Normal cells included PBLs, fibroblasts (Fibro), phytohemagglutinin stimulated PBLs (PHA), EBV-transformed B-cells (B-LCLs) derived from healthy human individuals.

* Treatment was at 41.8°C for 200 minutes and at 37°C for 4 hours (as described in Materials and Methods).

† Significantly different from control levels ($P < .01$) with Student's *t*-test.

bead separation method after adherence selection, a stimulated mixed effector cell population was divided into a $CD3^+$ T-cell population and a $CD3^-$ NK cell population. Purity of both cell populations was more than 95% as shown by FACScan analysis using CD3 and WT31 MoAbs as T-cell markers and CD16, CD56, and CD57 MoAbs as NK cell markers (Fig 2, legend). The percentage of γ/δ TCR^+ T cells was less than 5% in both cell fractions. The results derived from CML assays using unseparated (Fig 2A) and separated $CD3^+$ (Fig 2B) and $CD3^-$ (Fig 2C) effector cells against either untreated (37°C) or nonlethally heat-treated (heat shock [hs], 41.8°C for 200 minutes and 37°C for 4 hours) ES and K562 cells are shown in Fig 2. Here, we show that lysis of untreated and heat-treated K562 cells is mediated predominantly by the $CD3^-$ NK effector cells but not by $CD3^+$ T

Table 2. Flow Cytometric Analysis of HSP72 and MHC Class-I (W6/32 MoAb) Cell Surface Expression on ES Cells After a Single Nonlethal Heat Treatment Followed by Different Incubation Periods (0 to 96 Hours) at 37°C

Recovery Periods After 41.8°C* (no. of hours at 37°C)	MoAbs	
	HSP72	W6/32
0	4.2 ± 1.3 (62)	84.2 ± 2.6 (110)
1	15.7 ± 1.8 (98)†	82.4 ± 2.8 (103)
2	20.9 ± 1.7 (119)†	83.7 ± 2.4 (103)
3	28.3 ± 2.1 (120)†	73.3 ± 2.9 (91)†
4	31.4 ± 1.9 (117)†	70.5 ± 2.8 (94)†
5	26.4 ± 1.8 (104)†	68.9 ± 3.2 (98)†
6	27.4 ± 2.7 (109)†	72.9 ± 2.3 (98)†
8	27.5 ± 1.4 (100)†	84.0 ± 2.2 (118)
12	27.3 ± 1.5 (92)†	83.9 ± 2.4 (117)
24	26.6 ± 1.8 (89)†	83.7 ± 2.7 (110)
48	19.3 ± 1.3 (86)†	84.1 ± 2.2 (121)
72	19.7 ± 1.9 (90)†	83.4 ± 2.6 (119)
96	17.3 ± 2.1 (87)†	84.8 ± 2.9 (122)

Values shown are the percentage of positively stained ES cells ± standard deviation (SD) with the mean fluorescence intensity in parentheses. Anti-HSP27 (IgG1) and mouse IgG2a MoAbs were used as isotype-matched control antibodies for HSP72 and W6/32 MoAbs.

* Treatment was at 41.8°C for 200 minutes.

† Significantly different from control levels ($P < .01$) with Student's *t*-test.

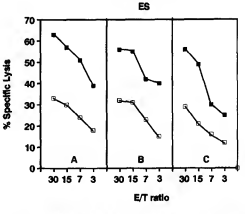

Fig 1. Increased sensitivity of nonlethally heat-shocked sarcoma cells to lysis by HLA-disparate effector cell populations. Cytotoxic activity of HLA-disparate effector cells (donors A, B, and C) showed no obvious differences in the lysis pattern. ^{51}Cr-labeled, heat-treated ES cells (■) were lysed to a much higher degree (about twofold), as compared with untreated ES target cells (□), by all three effector populations. This effect was titratable at varying E/T ratios ranging from 30:1 to 3:1. Each data point represents the mean value of three independent experiments; ± SD was always less than 10%. SR of untreated and heat-treated target cells was identical and below 20%. Similar lysis patterns were obtained with other sarcoma cell lines (HOS58, MG63) that were either untreated or nonlethally heat-treated (data not shown).

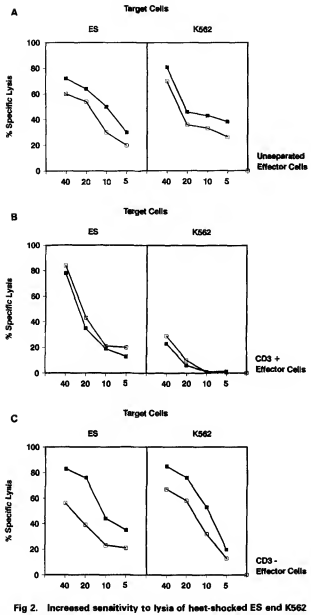

Fig 2. Increased sensitivity to lysis of heat-shocked ES and K562 cells is mediated by non-MHC-restricted, CD3⁻ NK cells, but not by CD3⁺ CTLs. Cytotoxic activity of (A) a mixed effector cell population (93%, CD3; 22%, CD16; 18%, CD56; 16%, CD57; 5%, γ/δ), (B) a CD3⁺ CTL-enriched (97%, CD3; 7%, CD16; 9%, CD56; 15%, CD57; 4%, γ/δ TCR), and (C) a CD3⁻ NK-enriched (5%, CD3; 43%, CD16; 45%, CD56; 32%, CD57; 1%, γ/δ TCR) effector cell population was tested separately to untreated (□) and heat-treated (■) K562 and ES target cells. Lysis of K562 cells, either untreated or heat-treated, was only observed with the CD3⁻ NK-enriched effector cell population, whereas the CTL-mediated lysis of K562 target cells was weak. The enhanced lysis of heat-treated ES cells was also mediated by the NK-enriched population. Untreated ES cells were lysed by both effector cell populations. E/T ratios ranged from 40:1 to 5:1. Each data point represents the mean value of three independent experiments; ± SD was always less than 8%. SR of untreated and heat-treated target cells was comparable and was always below 20%.

lymphocytes. The enhanced lysis of heat-shocked K562 cells is expressed more clearly by the NK population. The lysis of untreated ES cells is mediated by CD3⁺ T lymphocytes as well as by CD3⁻ NK cells. However, the enhanced sensitivity of heat-shocked ES cells is mediated predominantly by CD3⁻ NK cells.

Anti-HSP72 MoAb inhibits increased lysis of heat-treated K562 and ES cells. Antibody-blocking CML experiments using an NK-enriched effector cell population (phenotypic characterization, see the legend for Fig 3) were performed to define a possible recognition structure that is expressed on the cell surface of heat-shocked neoplastic cells. To exclude the effect of a transient MHC class-I downregulation on tumor target cells (4 hours after nonlethal heat shock), the lysis of untreated and heat-treated (41.8°C for 200 minutes) cells was compared after a 12-hour recovery period at 37°C (Table 2) when the MHC class-I expression has reached its initial level. Similar to the data presented in Figs 1 and 2 (important to note the 4-hour recovery period at 37°C), the lysis of heat-shocked ES (Fig 3A) and K562 (Fig 3B) cells followed by a 12-hour recovery period again was significantly higher as compared with the lysis of untreated (37°C) cells (Fig 3). Most important, this elevated lysis of heat-shocked neoplastic cells could be inhibited by preincubation of the target cells using HSP72-specific MoAb, whereas an isotype-matched control antibody directed against anti-HSP27 has no inhibitory effect. Further evidence that HSP72 is a relevant recognition structure for NK effector cells is given by the results obtained with the control tumor cell line MX-1 (Fig 3C). These cells do not express HSP72 molecules on their cell surface (Table 1), and their lysis was not enhanced after heat shock. Neither the HSP72 MoAb nor the isotype-matched control antibody (HSP27) had an inhibitory effect on the lysis of these cells after heat shock. Cytotoxic T lymphocyte (CTL) recognition of MX-1 tumor cells could be excluded, because blocking of MHC-restricted recognition by anti-MHC class-I MoAb or by anti-TCR MoAb has also no inhibitory effect. The anti-MHC class-I MoAb (W6/32) that is known to mask MHC class-I gene products[42,43] does not influence the lysis of heat-treated ES and K562 cells. A role of L243 MoAb (directed against HLA-DR region) that could mask MHC class-II gene products could be excluded, because no HLA-DR expression was detectable on the surface of ES and K562 cells. Preincubation of the effector cell population using WT31 MoAb (directed against α/β TCR⁺ T lymphocytes),[41] has no inhibitory effect on the lysis of heat-shocked neoplastic cells. These findings support the suggestion that the elevated lysis of heat-treated ES and of K562 cells is not mediated by MHC class-I–restricted T lymphocytes. As a control for CTL activity in the NK-enriched effector cells, we show that the lysis of allogeneic B-LCLs (Fig 3D) is inhibited by incubation of the effector cells using an anti-TCR–specific MoAb (WT31) or by blocking of MHC class-I molecules on the target cells using W6/32 MoAb.

DISCUSSION

In the present study, we provide direct evidence that a heat-inducible HSP72 cell surface expression is correlated with increased sensitivity of tumor cells against non-MHC–restricted NK cells. Recently, MHC-independent γ/δ TCR⁺, CD3⁺ T cells, which are considered to play a role in the defense against tumor cells,[44] are shown to recognize cell surface-expressed HSPs of the HSP60 and HSP70 fami-

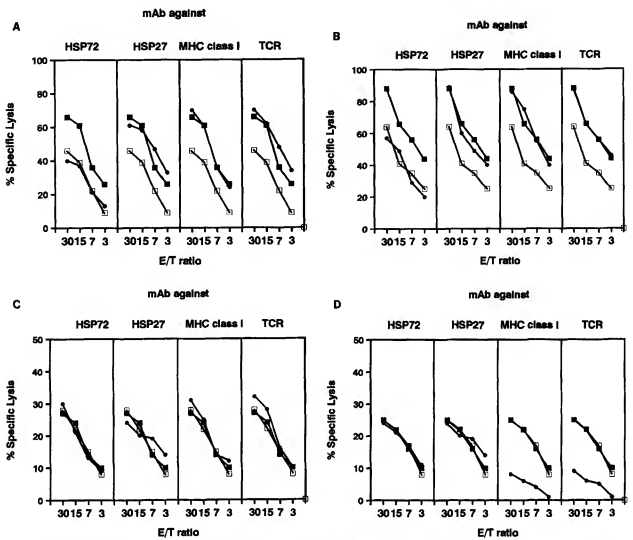

Fig 3. An HSP72-associated determinant acts as a recognition structure for NK-enriched (24%, CD3; 57%, CD16; 54%, CD56; 29%, CD57; 2%, γ/δ) effector cells on heat-shocked sarcoma cells. Effect of HSP72-specific (IgG1, HSP27-specific (IgG1, isotype-matched control), and MHC class-I–specific (W6/32; IgG2a) MoAbs on the lysis of heat-treated target cells, ie, ES (A), K562 (B), MX-1 (C), and allogeneic B-LCLs (D). (□), lysis of untreated (37°C) target cells; (▨) lysis of nonlethally heat-shocked (41.8°C for 200 minutes and 37°C for 12 hours) target cells; (●) lysis of heat-shocked target cells after antibody blocking. E/T ratios ranged from 30:1 to 3:1. Each data point represents the mean value of two independent experiments; ± SD was always less than 8%. SR of untreated and heat-treated target cells was identical and was below 20%. Lysis of ES and K562 tumor cells that express HSP72 (A and B) is inhibited using HSP72 MoAb; lysis of MX-1 carcinoma cells (C) and B-LCLs (D) that do not express HSP72 is not inhibited using HSP72 MoAb. The isotype-matched control antibody HSP27 (IgG1) has no inhibitory effect. Inhibition of the CTL-mediated lysis by MHC class-I–specific MoAb W6/32 (IgG2a) or by α/β TCR-specific MoAb WT31 (IgG1) has no inhibitory effect on the lysis of heat-shocked ES, K562, and MX-1 tumor cells (A, B, and C), whereas the lysis of heat-shocked allogeneic B-LCLs was inhibited by W6/32 and by WT31. Elevated lysis of other nonlethally heat-shocked sarcoma cells (HOS58, MG63 osteosarcoma cells) was also inhibited by HSP72 MoAb, but not by MHC class-I–specific (W6/32) or α/β TCR-specific (WT31) MoAbs (data not shown).

lies.[45,46] For the recognition of the heat-inducible HSP72 epitope on sarcoma cells, γ/δ TCR$^+$ T lymphocytes can be excluded, because the amount of γ/δ T lymphocytes within the stimulated effector cell population (as determined by FACScan analysis) was always below 5%. Cytotoxic activity mediated by CD3$^+$ lymphokine-activated killer cells[47,48] is also not very likely because, after separation of the effector cell population in a CD3$^+$ and a CD3$^-$ cell population, reactivity against heat-shocked tumor target cells was only found within the CD3$^-$ cell fraction. The CD3$^-$ cell population that was shown to be responsible for the increased lysis of heat-treated sarcoma cells and of classical NK target cells (K562) expresses NK markers CD16, CD56, and CD57. Lysis of untreated sarcoma cells is mediated by CD3$^-$ and CD3$^+$ effector cells. The transient reduction in the MHC class-I expression on tumor cells after heat shock is a possible explanation for the decreased lysis of heat-treated tumor cells by CD3$^+$ T lymphocytes, whereas there is no influence on the elevated lysis of heat-treated sarcoma cells mediated by CD3$^-$ NK effector cells. Preincubation of tumor as well as allogeneic target cells with MHC class-I–specific MoAb (W6/32), which is known to block the CTL-mediated cyto-

toxicity,[39,40] inhibits the lysis of allogeneic target cells but not the lysis of heat-shocked sarcoma and K562 cells. These findings are supported by blocking of α/β TCR$^+$ T cells using WT31 MoAb.[41] Again, only the lysis of allogeneic target cells, but not that of heat-shocked tumor and NK target cells, was blocked by this TCR-specific antibody. In contrast to the majority of cytotoxic responses of CTLs, which are known to be MHC-restricted, it has been shown that low levels of MHC class-I expression on tumor cells are associated with a higher susceptibility of lysis by NK cells.[42,49-51] Our observations that heat shock leads to a transient decrease in the MHC class-I expression could not be correlated with the elevated lysis of heat-shocked tumor cells that express HSP72. Therefore, we speculate that MHC-dependent[43] and MHC-independent[52,53] mechanisms of NK recognition have to exist. Despite the fact that certain HLA class-I alleles could be identified that act as negative signals for NK recognition on normal cells,[43] it is still not clear which target structures on tumor cells can act as positive recognition signals for NK cells. Our results derived from antibody-blocking studies using an HSP72-specific MoAb, suggest that a heat-inducible HSP72 epitope on sarcoma cells might act as a positive recognition structure for NK cells. This hypothesis is strongly supported by data derived from CML assays using heat-shocked carcinoma cells (MX-1) that showed no HSP72 cell surface expression after heat shock. The NK-mediated lysis of these target cells was not increased after heat shock. Therefore, we conclude that the increased sensitivity of heat-shocked sarcoma cells is caused by the HSP72 cell surface expression and is not a nonspecific effect of heat treatment. Furthermore, the increased lysis of heat-treated K562 cells and, to a lower extent, the lysis of untreated K562 cells were inhibited by HSP72 MoAb. These data led us to the hypothesis that heat-treated sarcoma cells and K562 cells have to share at least one recognition structure for NK cells, which seems to be closely associated with HSP72. Results derived from cold target inhibition studies support this hypothesis (data not shown). Recently, we showed that a 72-kD protein could be immunoprecipitated from the cellular membrane of different heat-treated tumor cells but not of normal cells by using the HSP72 MoAb.[21] These data are in line with observations of other groups that HSP72 cell surface localization was only found on affected cell types such as tumor cells,[54] human immunodeficiency virus-[19] and human T-cell lymphotrophic virus-infected cells,[55] mycobacterial-infected cells,[56] and affected retrocular fibroblasts obtained from patients suffering from Graves' ophthalmopathy.[18] In this context, it is important to note that HSPs are discussed to act as immunogenic determinants for different effector cell mechanisms.[57] Ullrich et al[58] and Srivastava et al[59] raised the hypothesis that HSPs homologous to members of the HSP90 and HSP70 family are recognized as tumor antigens in mice by CTLs. Carbohydrate side chains as antigenic determinants on these HSPs can be excluded, because none of them is glycosylated.[60] Tamura et al[61] showed that improved immunogenicity of Ha-*ras*–transfected fetal rat fibroblasts mediated by CD4 and CD8 double-negative T cells could be correlated with the presence of an HSP70 cognate protein on the cell surface of tumor cells.

In conclusion, our data show that nonlethal heat shock induces a cell surface expression of HSP72 that persists for at least 96 hours. This immunogenic epitope is selectively expressed on human sarcoma cells and can be correlated with NK recognition. Interestingly, normal cells and the MX-1 mammary carcinoma cell line fail to express this recognition structure. Additionally, heat treatment leads to a transient reduction in the MHC class-I expression. The HSP72 cell surface expression selectively on tumor cells could act as one possible recognition structure for an NK subpopulation. Our in vitro data that NK cells are relevant for recognition of heat-treated tumor cells are in line with recently published observations of an in vivo study that tumors are spontaneously infiltrated and killed by NK cells.[62] Furthermore, inhibition of metastasis has also been shown to correlate with the activity of NK cells.[63,64] NK-depleted mice seem to be more susceptible for rapid tumor growth after tumor transplantation.[65] Therefore, besides MHC-restricted effector mechanisms, such as CTL activities, non-MHC–restricted NK cells must also be considered as possible mediators for an antitumor immune response.

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